BIO-ASSAY OF THE SUBSTANCES OBTAINED FROM SARGASSUM TORTILE ON THE SETTLEMENT OF THE PLANULA OF CORYNE UCHIDAI

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BIO-ASSAY OF THE SUBSTANCES OBTAINED FROM *SARGASSUM TORTILE* ON THE SETTLEMENT OF THE PLANULA OF *CORYNE UCHIDAI*[1)]

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The neutral part of n-hexane extracts of the dried alga (3.5 Kg) was fractionated by the aid of column chromatography of silica gel for bio-assay, and a portion (302 mg) of the fractions showed a specific and intensive activity in favour of the settling and subsequent metamorphosis of swimming planulae of *Coryne uchidai*. By further purification, the active portion was separated into six compounds. On the basis of chemical and physical evidence, structure of B and A was deduced as δ-tocotrienol (I) and its epoxide (II). The deduction was unequivocally confirmed by the synthesis, in which DL-epoxide (II) was obtained by the reduction of dehydroepoxide (III). Since both epoxides have the same Rf on TLC and (II) shows M^+ and $(M-2)^+$ in its mass spectrum, the contamination of III in A is present. The remaining compounds have similar physical properties.

Hydrozoa are observed very often attaching to various algae. This association between epiphytic hydrozoa algae is formed by the settlement of hydrozoan larvae onto the algal thallus followed by the growth of the hydrozoan colony thereon (Kakinuma, 1960[a], 1960[b], Katô et al 1961, Nishihira, 1965[a]. 1965[b]. 1967). We have observed that most of epiphytic hydroids have their own preferable algae and that each association is established according to the algal preference of the settling larvae (Nishihira, 1967. 1968[a]., 1968[b]). This suggests that the algae might produce some specific chemical compound which induces the settling of swimming hydrozoan planulae. An athecate hydrozoan, *Coryne uchidai* is an example which proceeds to further differentiation after responding to the stimulation of attachment usually to *Sargassum*. The extract of *Sargassum tortile* was especially effective, and the active principle was water soluble and heat stable. These facts suggest that stimulation by preffered substratum, initiates differentiation of the planula of this species.

We searched for the active substances in *Sargassum tortile* by means of bioassay, using the swimming planulae of *Coryu uchidai*, and elucidated the structure of the

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active principle δ-Tochotrienol and its epoxide. The structure was unequivocally confirmed by synthesis and the ± epoxide (2) exhibited clear activity toward the settling of the swimming planulae.

MATERIALS AND METHODS

Fraction of Sargassum tortile: Dried and ground *Sarggassum tortile* (3.5 Kg), collected from the shallow water near the Asamushi Marine Biological Station of Tôhoku University, in October was extracted continuously for five days with 6 l n-hexane using a soxhlet apparatus. Evaporation of the solvent gave 31 g of viscous brown residue, which was then redissolved in ether, shaken with cold 0.1 N aqueous NaOH and separated into acidic and neutral parts. 22 g of the neutral part, thus obtained, was chromatographed on silica gel (1.1 Kg) and eluted successively with cyclohexane, cyclohexane-isopropyl ether (10:1), CH_2-Cl_2-MeOH (1:1), and finally with MeOH. Similar portions on TLC (thin layer chromatography) (SiO_2; $CHCl_3$) obtained from each elluent were combined and the neutral part was roughly divided into 20 fractions. The even numbered fractions were assayed as follows and 302 mg of active fraction 8 was obtained.

Bio-Assay of the even numberd fractions: The bio-assay was carried out from June 15 to June 17th (1971), since appearance of *Coryne uchidai* in field was limited strictly within a few weeks of this season in a year. Five larvae of *Coryne uchidai* were put in a petri dish containing 5 ml of filtered sea water. 10 mg of each even numbered fraction was dissolved in 1.25 mg of EtOH and one drop of the solution was added to the petri dish. The above experiment was repeated twice and almost the same results were obtained. For the control experiment, one drop of EtOH was added to the petri dish.

Purification of active fraction 8: Isolation of δ-tocotrienol (I) and its epoxide (s) (II, III (?)). The active fraction 8 showed the presence of six component on TLC (SiO_2; $CHCl_3$) with their Rf values of 0, 07, 0. 18, 0.23, 0, 32, 0.43, and 0.50 and denoted as fraction (compound) A (II, III), B (I), C, D, E, and F, respectively. These components were separated by preparative TLC. 302 mg of Fraction 8 was dissolved in 5 ml of $CHCl_3$ and each 0.5 ml of the solution was applied to a plate of Wakogel B-5 (20×20×0.1 cm) and developed with $CHCL_3$. The fluorescent charactor of each component against UV-lamp was effective for detection of the positions on the plate. SiO_2 bands of the same Rf values from all ten plates were gathered and extracted with $CHCl_2$- ether (1:1). In Table 2 were summarized the amounts of each component thus separated, together with their molecular formulae which were determined by high resolution mass spectrometry.

Fraction B (compound I, δ-tocotorienol), obtained in 89 mg a pale yellow oil showed $[\alpha]_D$ +22.15 ($CHCl_3$, C=3.1); IR (liquid film) 3350, 1660, 1610, 1220, 1150, 1105, 990, 930, 855 cm^{-1}; Mass m/e 396. 3026 $(M)^+$, $C_{27}H_{40}O_2$ requires 396. 3028; UV λmax 293 nm (ε=6180) in n-hexane, 300 nm (ε=2900) in EtOH.

Hydrogenation of I: A mixture of 123 mg of I and 80 mg of 5% pd/c in 2 ml of MeOH was shaken under hydrogen atmosphere at room temparature. After absorption of hydrogen ceased, the catalyst was removed and evaporation of the solvent gave 109 mg of hexahydro-I as a pale yellow oil. IR (liquid film) of hexahydro-1 showed 3350, 1610, 1220, 1150, 850 cm^{-1}; Mass 402.3477 (M, $C_{27}H_{46}O_3$ requires 402. 3498); UVλ_{max} 300 nm (ε=6470) in n-hexane, 300 nm (ε=3500) in EtOH.

Acetylation of Hexahydro-I. A mixture of 14 mg of hexahydro-I, 5 mg of Ac ONA, and 0.5 mg of Ac_2O in 5 ml of benzene was refluxed overnight. The reaction mixture was diluted with water, and dried over $MgSO_4$. Evaporation of the solvents gave 14.8 mg of pale yellow oil, hexahydro-I-acetate. IR showed 1760, 1610, 1200, 1010 cm^{-1}; NMR (nuclear magnetic resonance) (T-60, CCl_4) 1.89 and 2.75 (2H each, *t*, 7Hz -CH_2-CH_2-ph), 2.16 and 2.19 (3H, each, s, methyls on aromatic ring and acetyl group), 6.66 (2H, bs, aromatic protons); Mass m/e 444 (M).

Fraction A (II, III): Fraction A was obtained in 22 mg from the active fraction 8 as a pale yellow oil, IR 3350, 1610, 1218, 1160, 1100, 850 cm^{-1}; NMR (T-60, CCl_4) 1.22 (9H, s, -O-Mex3), 1.58 (6H, s, C=C-Me), 2.1 (3H, s, aromatic Me), 2.62 (m, protons of - methylene and epoxide), 5.1 (2H, m, olefinic-H), 412.3 (M of II) (relative intensity of 410 and 412 3:10, 177.0 ($O_{11}H_{13}O_2$), 175.0 ($C_{11}H_{11}O_2$, as a base peak), 137.0 ($C_8H_9O_2$).

OBSERVATIONS AND CONCLUSIONS

Bio-Assay of active substances

The neutral part of n-hexane extracts of the dried algae was fractioned with column and thin layer chromatography of sillica gel into 20 fractions. The even numbered fractions screened for bioassay against the induction of settling of the swimming larvae of *Coryne uchidai*: Table 1 shows how the swimming planulae responded; different fractions were added to the petri dishes containing planulae. Evidently, Fraction 8 showed a specific and powerful activity toward the settling and subsequent metamorphosis of the planula. In this case all the planulae settled within 30 minuites, and completely metamorphosed, with the formation of tentacle buds, after one day and grew up into polyps after two days. On the other hand, in the controls the planulae still remained in the crawling condition even after two days. Crawling is a condition where the swimming of the planulae becomes slow, and usually appears before settling.

Separation and identification of active substances

From 3.5 Kg of the dried algae 306 mg of 8 Fraction were collected. From this material six fractions were separated as shown in Table 2. From the molecular

Table 1
Bioassay of even numbered fractions of *Sargassum tortile*

Fraction No.	Hours after treatment						
	0	0.5	1.5	3.5	18.5	43	48
2	m	m	m	m	cl	s	s
4	m	cl	cl	cl	cl	s	cytl
6	m	m	m	cl	cytl	—	—
8	m	s	s	s	s	b	p
10	m	cl	cl	s	cytl	—	—
12	m	m	m	m	m	cl	cytl
14	m	m	m	cl	s	el	cytl
16	m	m	m	cl	cl	cl	s
18	m	m	m	cl	cl	cytl	s
20	m	cl	s	s	s	cl	—
Control	m	m	m	m	m	—	cl

Key to abbreviations: m-swimming b-tentacle bud formation cl-crawling
p-polyp formation s-settling el-elongation cytl-cytolysis

Table 2
Separation of Fr. 8 in Table 1

Fraction (Compound)	Wt. (mg)	Mol. Formula (M^+)
A	22	$C_{27}H_{40}O_3$ (412.3)
B	89	$C_{27}H_{40}O_2$ (396.3)
C	24	$C_{27}H_{40}O_3$ (412.3)
D	11	$C_{27}H_{38}O_3$ (410.2)
E	6	$C_{27}H_{40}O_2$ (396.3)
F	18	$C_{30}H_{42}O_2$ (434.3)

formulae and other physical evidence, all these components except F appeared to be structurally similar and hence, earlier chemical investigation was focused on fraction B (Compound 1) which was more plentiful.

During catalytic hydrogenation 1 three moles of hydrogen were consumed to give hexahydro-1, which produced a monoacetate on acetylation under usual condition. Both compounds, hydrogenation 1 and hexahydro 1, showed λ_{max}^{EtOH} at 300 nm in the UV spectra, indicating the presence of oxygenated aromatic ring.

In table 3 and 4, the NMR and high resolution mass spectral data were summarized as well as the possible group deduced therefrom. Presence of farnesyl group in hydrogenation 1 was suggested by physical evidence where three olefinic protons and four olefinic methyls were observed in the NMR spectrum and at the same time, relatively strong fragment ions were detected in the mass spectrum at

Table 3
NMR of II and Hexahydro-II

II	Hexahydro-II
—	0.87 (6H, d, 6.5) $-CH(CH_3)_2$
—	near 1.0 $>CH(CH_3)$ ×2
1.22 (3H, s) $>C(CH_3)-O-$	1.20 (3H, s) $>C(CH_3)-O-$
1.56 (9H, s) $C=C<^{CH_3}$ cis	—
1.64 (3H, s) $C=C<^{CH_3}$ trans	—
2.04 (3H, s) arom $-CH_3$	2.04 (3H, s) arom $-CH_3$
1.69, 2.62 (2H, each, t, 7) Ph-CH_2-CH_2-■	1.69, 2.26 (2H, each, t, 7) Ph-CH_2-CH_2-■
5.06 (3H, bm) C=CH-	—
6.20 (1H, d, 2.3) arom -H	6.20 (1H, d, 2.3) arom -H
6.32 (1H, d, 2.3) arom -H	6.32 (1H, d, 2.3) arom -H

Table 4
Fragment ions of II and hexahydro-II

II		Hexahydro-II	
Fragment ion (Composition)	relative intensity	Fragment ion (Composition)	relative intensity
396 (M^+)	27%	402 (M^+)	100%
192 ($C_{12}H_{16}O_2$)$^+$	19	192 ($C_{12}H_{16}O_2$)$^+$	10
177 ($C_{11}H_{13}O_2$)$^+$	19	177 ($C_{11}H_{13}O_2$)$^+$	56
137 ($C_8H_9O_2$)$^+$	46	137 ($C_8H_9O_2$)$^+$	100
69, 137, 205 (strong)		—	

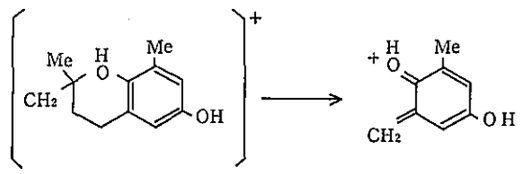
m/e 69 ($C_5H_9^+$), 137 ($C_{10}H_{17}^+$), and 205 ($C_{15}H_{25}^+$) due to allylic bond fissions of the farnesyl group.

The sequence of ph. $CH_2 \cdot CH_2$- was confirmed by decoupling experiments. Irradiation at 2.62 ppm due to α-methylene adjacent to phenyl group caused the triplet at 1.69 (β-methylene) to change into a clear singlet.

In the NMR spectrum of hydrogenation 1, two aromatic protons at 6.20 and 6.32 ppm exhibited a meta coupling with 2.3 H_8, while three protons appearing at 2.04 ppm were designated as an aromatic methyl. As described previously, hexahydro-1 afforded a monoacetate, in which no proton on the carbon bearing the acetoxy group could be detected. Similarly, in the spectra of 1 and its hexahydro

derivative, no proton geminal to OH group could be observed. Hence, the OH group should be located on the aromatic ring and this deduction is also supported by UV spectra.

Based on the above mentioned results, the strong common peaks at m/e 192, 177, and 137 in the mass spectra of both compounds were explained by the structure depicted in Fig 1.



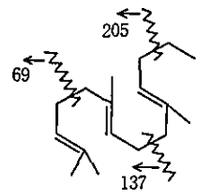
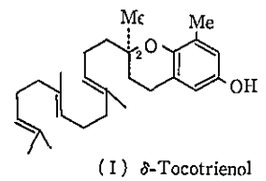


Fig. 1 Possible structures of fragment ions in Table 4

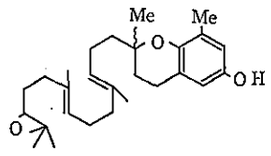
Consideration of all the evidence described above leads to the postulation that compound 1 is δ-tocotrienol (1), which has been isolated by PENNOCK et al. from Hevea Latex. Indeed, the IR spectrum of our compound is identical with that reported by PENNOCK et al. and also IR and NMR spectra in solution were completely in accord with those of dl-δ-tocotrienol synthesized by ourselves.

We deduced the absolute configuration of our compound based upon its optical rotation, although PENNOCK did not describe on it. Our δ-tocotrienol possesses one asymmetric carbon and has $[\alpha]_D$+22.15°. It is known that methly group at C_2 position of all natural tocopherols has α- configuration and all show positive optical rotation independently of the kind of their side chain.

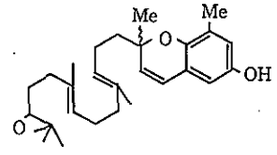
As described in Table 2, in addition to fraction B (compound 1) the active material inducing settlement contained some other constituents, viz., A, C, D, E



(I) δ-Tocotrienol



(II)



(III)

Fig. 2 Final result of the chemical structures

and F. Among them the following evidence indicated that fraction A is mainly composed of compound (II) although contamination with small amounts of dehydro derivative (III) can not be completely ruled out at present.

Spectral data of A are alike with those of I excepting the following details: In the NMR spectrum of A the number of protons and methyls on olefinic double bonds are relatively smaller than that of 1 while the signals at 1,2 ppm increased due to methyls geminal to oxygen function, and multiplets at 2,6 ppm, assignable to protons of α-methylene and an epoxide also increased. This evidence suggests that an oxygen function was introduced into the terminal double bond of farnesyl moiety of 1. In the high resolution mass spectrum of A, the molecular ion of II was observed at 412.3 and at the same time a weak peak at 410.2 was also detected which corresponds to $C_{27}H_{33}O_3$ (III). The fragment ion at m/e 175 appeared as a base peak accompanying a prominent peak at m/e 177 ($C_{11}H_{13}O_2$), the plausible structures of which are shown in figure 1.

Finally, the NMR of A is almost identical with that of the synthetic epoxide (II) and all the prominent peaks in the mass spectrum of A are confirmed to overlap with those of synthetic II. In the mass spectra of both synthetic II and III, the base peak was observed at m/e 175.

Although other fractions, C, D, and E were not studied in details, mass spectra of these compounds showed prominent peaks at m/e 177 and 175, indicating the presence of chromane moiety.

In conclusion, the presence of δ-tocotrienol (I) and its related epoxide (s) (II, III (?)) have been ascertained from the active portion of *Sargassum tortile*, that is the fraction which induces the settling of swimming planulae of *Coryne uchidai*. It was found that our synthetic materials, especially both epoxides (II and III) are effective toward the assay using swimming larvae of *Coryne uchidai* as shown in Table 5.

It is clear from this experiment that δ-tocotrienol (I) induced settling and polyp formation only in some planulae. On the other hand, with DL epoxide (II) and dehydroepoxide (III) all planulae settled and differentiated within 72 hrs. Of the 30 planulae used in 3 controls. 3 swam only, 13 crawled only, 8 settled and 6 attached in 72 hrs. None showed any differentiation. During the bioassay of the experiment described above, we found a very interesting phenomenon.

The metamorphosis from the planula into the polyp which was promoted by the epoxide was stopped at an early stage. After that, the polyps degenerated day

Table 5

Bio-Assay of synthetic I, II, and III toward the planulae of *Coryne uchidai*

Time (h)	12~24						48						72					
Stage[a]	m	cl	s	a	b	p	m	cl	s	a	b	p	m	cl	s	a	b	p
δ-Tocotrienol (I)[b]		6	2		2			7			1	2			7[d]			3
Control[c]	2	8						10						10				
Epoxide (II)[e]			3	7					5			5					5	5
Epoxide (II)[f]		3	4	3				2		5	1	2					7	3
Control[c]		5	5					5	5					2	3	5		
Dehydro epoxide (III)[g]		5	2	3					1	4		5				4		6
Control[h]	4	2	2				4	2	4				3	1	5	1		

a) Abbreviations; m=swimming, cl=crawling, s=settling, a=attaching, b=formation of tentacle bud, and p=formation of polyps.
b) One drop (0.05 ml) of ethanol solution containing 15 mg of dl-(1) in 1 ml of ethanol was added to ten planulae in 20 ml of sea water. The values show the number of larvae in different stages.
c) One drop of ethanol containing no material was added under same conditions.
d) All of the 7 planulae died accompanying cytolysis.
e) One drop of ethanol solution containing 30 mg of II was added to ten planulae in 20 ml of the sea water.
f) A qurater of one drop of the above original solution was used.
g) One drop of ethanol solution containing 30 mg of epoxide (III) in 1 ml of EtOH was droped on filter paper and the solvent was evaporated. The filter paper was put in 20 ml of sea water containing ten larvae.
h) Filter paper without material was put in sea water.

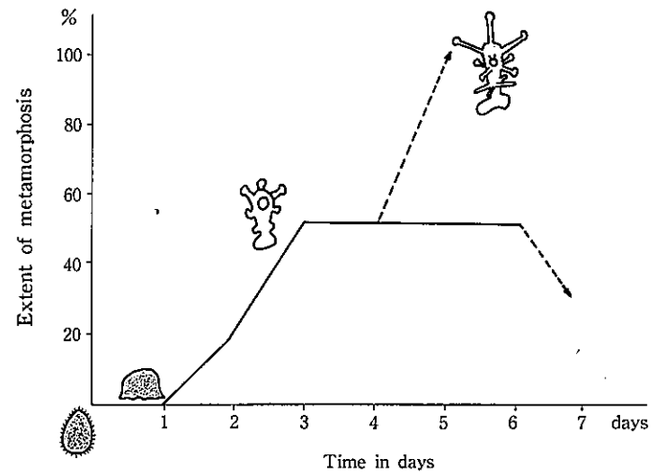


Fig. 3 Inhibition of the polyp formation by the epoxide (II)

by day. However, if at this stage, the uncompleted polyps stopped growing and were returned to normal sea water, they became completely normal within one day, (shown in Fig. 3).

These observations suggest that the epoxide II exerts control of metamorphosis, inducing differentiation but inhibiting complete polyp formation. These effects may depend on concentrations. It is interesting that the active substance resembles the insect moulting hormone, ecdysin, in its chemical structure.

The effect of the algal substances which control the metamorphoses of planulae of *Coryne uchidai*, was observed not only in one isolated substance but also in various components of the algae in geometric progression. The next problems are to find which part of the molecule is most effective and also find the minimal concentration for the appearrance of activity.

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